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The ALP-Enigma Protein ALP-1 Functions in Actin Filament Organization to Promote Muscle Structural Integrity in *Caenorhabditis elegans*

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Mutations that affect the Z-disk–associated ALP-Enigma proteins have been linked to human muscular and cardiac diseases. Despite their clear physiological significance for human health, the mechanism of action of ALP-Enigma proteins is largely unknown. In *Caenorhabditis elegans*, the ALP-Enigma protein family is encoded by a single gene, *alp-1*; thus *C. elegans* provides an excellent model to study ALP-Enigma function. Here we present a molecular and genetic analysis of ALP-Enigma function in *C. elegans*. We show that ALP-1 and α-actinin colocalize at dense bodies where actin filaments are anchored and that the proper localization of ALP-1 at dense bodies is dependent on α-actinin. Our analysis of *alp-1* mutants demonstrates that ALP-1 functions to maintain actin filament organization and participates in muscle stabilization during contraction. Reducing α-actinin activity enhances the actin filament phenotype of the *alp-1* mutants, suggesting that ALP-1 and α-actinin function in the same cellular process. Like α-actinin, *alp-1* also interacts genetically with a connectin/titin family member, *ketn-1*, to provide mechanical stability for supporting body wall muscle contraction. Taken together, our data demonstrate that ALP-1 and α-actinin function together to stabilize actin filaments and promote muscle structural integrity.

INTRODUCTION

Muscle generates contractile force and is required for processes such as locomotion, respiration, beating of the heart, and peristalsis. How muscle cells become specified, assemble the contractile machinery, and generate force have been extensively studied (Perry, 1996; Arnold and Braun, 2000; Gregorio and Antin, 2000; Clark *et al.*, 2002; Pownall *et al.*, 2002). However, little is understood about mechanisms involved in maintaining muscle structural integrity. Muscular dystrophies are a group of hereditary muscle diseases in which muscle integrity is compromised. Patients appear normal at birth, but are characterized by progressive muscle weakness. Identification of molecular mechanisms underlying muscle maintenance will increase our knowledge of muscle biology and provide insights into these and other diseases.

In striated muscle, Z-discs are important functional sites for muscle cytoarchitecture, force transmission, and signal transduction (Clark *et al.*, 2002). Z-discs define the lateral boundaries of the sarcomere and constitute anchoring sites for actin filaments. α-Actinin, the predominant protein component of Z-discs, functions to cross-link and organize actin filaments (Blanchard *et al.*, 1989). Genetic studies in *Drosophila* have shown that α-actinin–deficient mutants complete embryogenesis, but exhibit progressive muscular paralysis and die within a few days of hatching (Fyrberg *et al.*, 1990; Roulier *et al.*, 1992; Fyrberg *et al.*, 1998). Although the α-actinin deficient flies exhibit very severe phenotypes, the observation that they retain some muscle function suggests that other Z-disk–associated proteins may also participate in anchorage and stabilization of the actin filaments.

The ALP (α-actinin–associated LIM protein)-Enigma proteins are a group of evolutionarily conserved proteins that are prominently localized at the Z-disk and other sites of actin filament anchorage (Te Velthuis *et al.*, 2007). ALP-Enigma family members feature a single PDZ domain in the N-terminus and either one or three LIM domains in the C-terminus; both the PDZ and LIM domains are protein-binding interfaces. The PDZ domain, a ~80–120 amino acid β-barrel structure, is found in a variety of signaling molecules (Doyle *et al.*, 1996; Harris and Lim, 2001; Au *et al.*, 2004). Notably, in the ALP-Enigma family, the signature sequence of the PDZ domain, Gly-Leu-Gly-Phe, is replaced with Pro/Ser-Trp-Glu-Phe (Doyle *et al.*, 1996; Guy *et al.*, 1999). This sequence is located in the PDZ domain's binding groove, which is an important site for interaction with binding partners (Doyle *et al.*, 1996; Guy *et al.*, 1999), suggesting that the ALP-Enigma family may have different protein target specificities than other PDZ-containing proteins. LIM domains are double zinc-finger modules and have been shown to mediate diverse biological processes (Schmeichel and Beckerle, 1994; Kadrmas and Beckerle, 2004). Together, the multidomain structure of the ALP-Enigma family suggests a role for these products in protein targeting and protein complex assembly.

Seven ALP-Enigma family members have been identified in vertebrates: ALP, RIL, CLP36, Mystique, Enigma, ENH, and Cypher. ALP-Enigma proteins are highly enriched in cardiac and skeletal muscles (Wang *et al.*, 1995; Kuroda *et al.*, 1996; Xia *et al.*, 1997; Faulkner *et al.*, 1999; Kotaka *et al.*, 1999, 2001; Pomies *et al.*, 1999; Zhou *et al.*, 1999; Huang *et al.*, 2003; Niederlander *et al.*, 2004), and genetic analyses have re-

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vealed important roles for members of this family in muscle. Most ALP-Enigma proteins have been shown to interact with α-actinin (Xia *et al.*, 1997; Faulkner *et al.*, 1999; Pomies *et al.*, 1999; Zhou *et al.*, 1999; Kotaka *et al.*, 2000; Nakagawa *et al.*, 2000; Niederlander *et al.*, 2004; Schulz *et al.*, 2004; Jani *et al.*, 2007). In vitro studies revealed that chicken Alp enhances the cross-linking of actin by α-actinin (Pashmforoush *et al.*, 2001); however, their functional relationship in vivo is not understood. Targeted disruption of murine Alp results in right ventricular cardiomyopathy (Pashmforoush *et al.*, 2001). Mice that lack Cypher display congenital myopathy and die from failure in multiple striated muscles at the onset of muscle use (Zhou *et al.*, 2001). These studies demonstrate that ALP-Enigma proteins are critical for muscle function and may participate in muscle stabilization. Still, details regarding how this family of proteins influences muscle function are unclear.

Caenorhabditis elegans is a powerful model system in which to approach the genetic basis of muscle structure and function. With conserved muscle components, a similar sarcomere structure, and a wide variety of genetic tools (Waterston, 1988; Moerman and Fire, 1997), *C. elegans* is a fast and efficient system that complements vertebrate studies of muscle proteins. We previously reported that *C. elegans* contains a single gene, *alp-1*, that encodes the entire ALP-Enigma family of proteins and that ALP-1 proteins are highly enriched in the musculature (McKeown *et al.*, 2006). Here we have extended these studies by characterizing the subcellular localization of ALP-1 in *C. elegans* body wall muscle, analyzing *alp-1* mutants, and defining the relationship between ALP-1 and α-actinin. Our studies suggest a model in which ALP-1 proteins function together with α-actinin to stabilize actin myofilaments, thus promoting muscle structural integrity.

MATERIALS AND METHODS

Nematode Strains and Genetics

C. elegans strains were grown under standard conditions (Brenner, 1974) at 22°C. Bristol N2 was used as wild type. N2 and *alp-1(ok820)* strains were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). The *alp-1(tm1137)* deletion mutant was provided by Dr. Mitani (National BioResource Project, Tokyo, Japan). The α-actinin mutant *atn-1(ok84)* was provided by Dr. Robert Barstead (Oklahoma Medical Research Foundation, Oklahoma City, OK). At least six independent outcrosses were performed for the *alp-1* mutant strain before the analysis was conducted.

Western Immunoblot

Worm lysates were prepared by collecting worms of mixed stages and homogenizing them in RIPA lysis buffer. Protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Lysates were then electrophoresed through 10 or 15% SDS-polyacrylamide gels and immunoblotted by standard methods (Towbin *et al.*, 1979). Primary antibodies used were as follows: rabbit affinity-purified anti-ALP-1A (B74, 1:1000); rabbit affinity-purified anti-ALP-1PDZ (B78, 1:1000); anti-actin (C4, ICN Biomedicals, Costa Mesa, CA, 1:6000); anti-myo-2 (9.2.1, 1:7000; Miller *et al.*, 1986); anti-tubulin (12G10, Developmental Studies Hybridoma Bank, Iowa City, IA, 1:10,000). Horseradish peroxidase–linked ECL anti-rabbit or anti-mouse IgG (Amersham Pharmacia, Piscataway, NJ) were used as secondary antibodies.

Rabbit polyclonal antisera B74 and B78 were generated (Harlan Bioproducts, Stoughton, MA) using peptide antigens (PVPSNPPPSNVRSWW) and peptide antigens (MARSDRRTPWGFGVTEA) corresponding to the last 15 amino acids and the residues (8-24) of the predicted ALP-1A protein. The sera were affinity-purified against the peptide using standard procedures (Pierce, Rockford, IL).

RNA Interference

RNA interference (RNAi) experiments were performed as previously described (Kamath *et al.*, 2001). In the established RNAi library (Geneservice, Cambridge, United Kingdom), two clones, IV-4D11 and IV-4D13, are found to affect *alp-1*. The clone IV-4D11 is designed to knock down *alp-1*; however, it only affects the *alp-1d* transcript (our unpublished data). The other clone IV-4D13 expresses the double-strand RNA (dsRNA) fragment covering a region of the *alp-1* gene shared among all ALP-1 isoforms and also another gene *T11B7.5*, which is within an *alp-1* intron. Thus, interference experiments using clone IV-4D13 should knockdown all *alp-1* transcripts as well as *T11B7.5* transcript. Because the *alp-1(ok820)* mutant deletes the entire *T11B7.5* gene and yet still displays normal muscle function (our unpublished data), *T11B7.5* function is presumably not required for proper muscle contraction. Our studies used the clone IV-4D13 to knockdown the *alp-1* gene function and refer it to *alp-1* RNAi clone.

Muscle Contractility Assays

A pumping assay was performed to examine the contractility of pharyngeal muscle. Individual adults that laid their first egg within 24 h were scored visually for pharyngeal pumping using a Zeiss Stemi 2000 dissecting microscope (Thornwood, NY). For each animal, the movement of the grinder was counted for three 10-s time periods.

A thrashing assay was performed to examine the contractility of body wall muscle. Worms that laid their first eggs within 24 h were picked into a drop of 10 μl M9 buffer on a glass slide and allowed to recover from the transfer for 1 min. Thrashing movements were then counted for 1 min. Animals that ceased to thrash for more than 5 s were excluded from the analysis.

Fluorescence Microscopy

Indirect immunostaining was performed using a whole-mount fixation method as described (Finney and Ruvkun, 1990; McKeown *et al.*, 2006). Primary antibodies used included: anti-α-actinin (MH35, gift from R. Waterston, University of Washington, 1:250), anti-vinculin (MH24, Developmental Studies Hybridoma Bank, undiluted), anti-ALP-1 (B78, 1:200), and anti-myosin (5–6, Developmental Studies Hybridoma Bank, 1:100). Alexa 488– or Alexa 568–conjugated secondary antibodies were used (Molecular Probes, Eugene, OR).

For phalloidin staining, young adults were collected in S-medium, fixed in 2% formaldehyde for 10 min at RT, and permeabilized in 100% acetone at RT for 5 min, after a serial dilution of acetone. Worms were then washed three times with PBS and incubated with Alexa 488–conjugated phalloidin (Molecular Probes, 1:500 dilution) at RT for 40 min. After washing four times in PBS, samples were mounted to visualize actin filaments.

All images were acquired using an Olympus FV300 confocal imaging system (Melville, NY) and processed using ImageJ (NIH; http://rsb.info.nih.gov/ij/) and Adobe Photoshop and Illustrator software (San Jose, CA).

RESULTS

Generation of anti-ALP-1–specific Antibodies

A single gene *alp-1* encodes all the ALP-Enigma protein isoforms of *C. elegans*; *alp-1* is predicted to produce one ALP-like and three Enigma-like isoforms (McKeown *et al.*, 2006; Figure 1, A and B). In an effort to dissect the function of ALP-Enigma proteins in *C. elegans*, we raised two ALP-1–specific antibodies: B74 and B78 (Figure 1B). The B74 antibody is generated against the ALP-1A–specific region. In Western analysis, B74 recognized a band of ~46 kDa, corresponding to the ALP-1A isoform (Figure 1C). The B78 antibody is directed against the ALP-1 shared PDZ domain (Figure 1B) and thus should recognize the ALP-1A, B, and D isoforms. Western blot analysis showed B78 reacts with three proteins corresponding to ALP-1A, B, and D (Figure 1D). By Western blot analysis, ALP-1A and B migrate as expected based on their sequences, whereas ALP-1D migrates much slowly than predicted. The specificity of both antibodies has been shown by using the *alp-1* mutants (see below). Although we previously reported that the *alp-1* gene produces four alternatively spliced variants (McKeown *et al.*, 2006), we have not been successful in confirming expression of ALP-1C by either Western blot analysis or sensitive RT-PCR using *alp-1c*–specific primers (data not shown). Previous evidence for the expression of four transcripts came from Northern analysis, and we currently believe that there is a nonspecific cross-hybridizing RNA species that migrates at the same region predicted for *alp-1c*. Alternatively, if ALP-1C does exist, it must be expressed at very low levels or in a tissue- or temporally restricted pattern that makes detection in unstaged worm populations difficult.

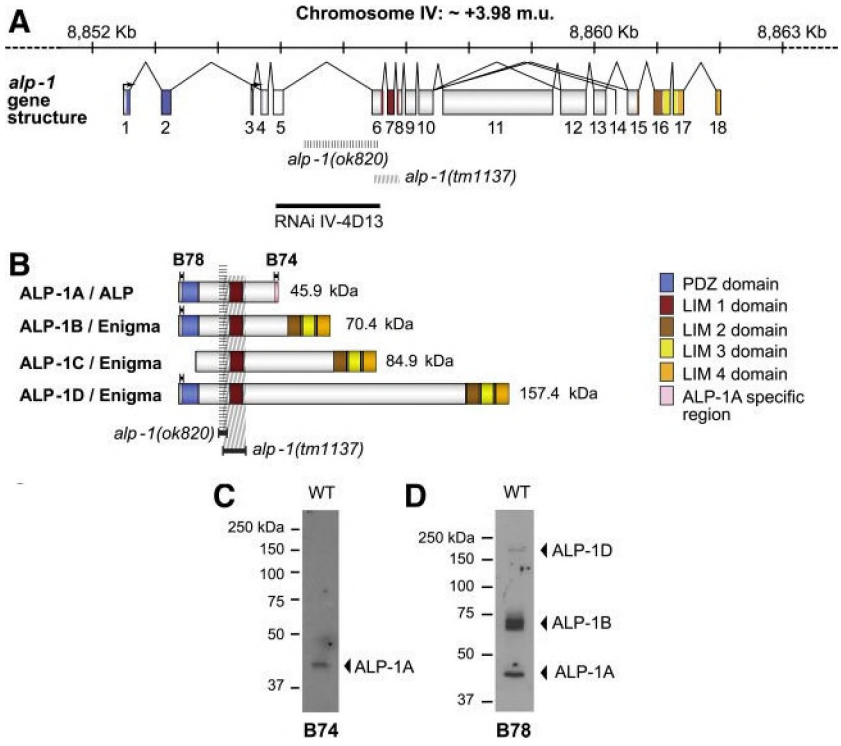

Figure 1. Schematic representation of the *alp-1* gene and gene products. (A) The genomic organization of the *alp-1* gene. Exon numbers and alternative splice points are indicated. Colored boxes indicate motifs that are identified in B. Hatched bars here as well as in B indicate the extent of the deletions in the *alp-1(tm1137)* and *alp-1(ok820)* mutants. The black bar indicates the dsRNA fragment expressed in the RNAi clone IV-4D13 (*alp-1* RNAi), which is used to knockdown all *alp-1* transcripts. (B) Four ALP-1 isoforms are suggested to be produced from the *alp-1* gene in previous report (McKeown *et al.*, 2006). Locations of the peptide antigens used for antibodies production are labeled B74 (anti-ALP-1A antibody) and B78 (anti-ALP-1PDZ antibody). The B74 peptide antigen, which is labeled by the pink color block, is specific to the ALP-1A and is not present in other ALP-1 isoforms. The position and identity of motifs present in the ALP-1 isoforms are indicated by colored blocks. (C and D) Western immunoblots of worm lysates show that (C) the B74 antibody specifically recognizes ALP-1A isoform and (D) the B78 antibody reacts with isoforms ALP-1A, B, and D.

ALP-1 Specifically Colocalizes with α-Actinin at Dense Bodies

To gain insight into the functions of ALP-1 proteins, we used the anti-ALP-1 antibodies to examine their expression pattern and subcellular localization. Immunostaining of wild-type worms using B74 or B78 revealed that endogenous ALP-1 proteins are highly expressed in the body wall muscle throughout postembryonic development (Figure 2A). In addition, B78 (against ALP-1A, B, and D) also detects ALP-1 proteins at the apical and basal surfaces of the pharynx, another musculature structure in the worm, but at a very low level of expression (Figure 2B). These results suggest that ALP-1 proteins function in muscle cells and probably primarily in the body wall muscle.

We performed double labeling with other muscle components and analyzed confocal microscope optic sections to define the subcellular localization of endogenous ALP-1 proteins in body wall muscle. Within body wall muscle, anti-ALP-1 staining appeared in a periodic punctate array that was identified as dense bodies by colabeling with an α-actinin antibody (Figure 2C). It should be noted that in body wall muscle, using anti-ALP-1 antibodies we did not observe any nuclear staining, such as that reported for the ALP-1::GFP translational reporter (McKeown *et al.*, 2006; see *Discussion*). In *C. elegans* body wall muscle, the contractile units attach to the muscle membrane at dense bodies. The dense bodies also serve to cross-link the thin filaments and are thus functionally equivalent to the Z-discs of vertebrate striated muscle (Waterston, 1988). Proteins found in dense bodies have been shown to distribute nonuniformly within the dense body. For example, vinculin is found at the base of the dense bodies near the membrane, whereas α-actinin is localized to the more cytoplasmic portion of dense bodies (Francis and Waterston, 1985). To determine in which subregion ALP-1 proteins are located, we colabeled body wall muscle with antibodies directly against ALP-1(B78) and vinculin. Double labeling with anti-ALP-1 and anti-vinculin antibodies revealed both proteins are localized at dense bodies, but ALP-1 is absent from muscle cell–cell junctions (dense plaques) that contain vinculin (Figure 2D). High magnification views of the dense bodies revealed that ALP-1 proteins colocalize with α-actinin (Figure 2C′), but only have limited overlap with vinculin (Figure 2D′). These data indicate ALP-1 and α-actinin colocalize within the dense body in a region that is spatially distinct from the basal, membrane proximal zone that accumulates vinculin (Figure 2E).

Proper Localization of ALP-1 at Dense Bodies Is Dependent on α-Actinin

To better understand the functional relationship between ALP-1 and α-actinin, we evaluated the localization of ALP-1 in α-actinin mutants. The α-actinin mutant *atn-1(ok84)*, kindly provided by R. Barstead, contains a 1.1-kb deletion and is predicted to be a null allele of the sole α-actinin gene (*atn-1*) in *C. elegans* (Barstead *et al.*, 1991). Although *atn-1(ok84)* mutants are viable and motile, their dense bodies are expanded slightly compared with wild type, but still retain a periodic punctate appearance as shown by vinculin staining (Figure 3; Ono *et al.*, 2006). In contrast to vinculin staining, the location of ALP-1 proteins in *atn-1(ok84)* mutants was not restricted to dense bodies and the pattern of ALP-1 proteins was more continuous instead of punctate (Figure 3B). ALP-1 proteins still associate with thin filaments of the cytoskeleton in *atn-1(ok84)* mutants, as suggested by the relationship of linear staining with the anti-ALP-1 antibody to the dense body (Figure 3B) and the colocalization of ALP-1 and actin (data not shown). Additionally, we occasionally detect ALP-1 proteins at dense plaques (muscle–muscle junctions) in the *atn-1* mutants (Figure 3B, arrow), a situation not found in wild-type worms (Figure 2D, arrow). Our data demonstrate that α-actinin is required for proper dense body localization of ALP-1 and confirms that vinculin retains some capacity to accumulate at dense body puncta in the absence of α-actinin.

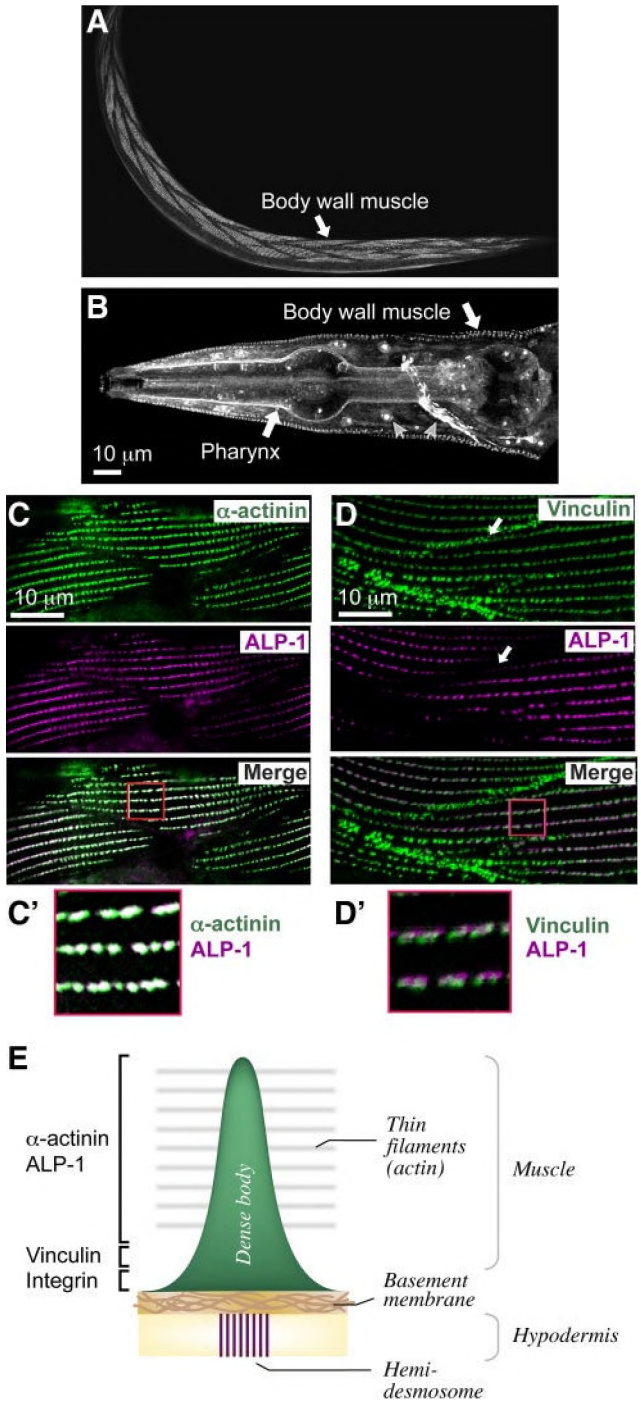

Figure 2. ALP-1 is colocalized with α-actinin, but not vinculin within the muscle dense bodies. (A) Using B74 antibody, endogenous ALP-1 proteins are detected in body wall muscle in strips along the longitudinal axis of the worm. (B) The ALP-1 proteins are localized at the apical and basal surfaces in the pharynx using B78 antibody. Note that the nuclear-like staining and neuron-ring staining (gray arrowheads) around the pharynx are nonspecific labeling by the B78 antibody. (C and D) The body wall muscle from wild-type worms are labeled with (C) α-actinin (green) and ALP-1 (magenta), or (D) vinculin (green) and ALP-1 (magenta). (C') An enlargement of C shows that α-actinin (green) specifically colocalizes with ALP-1 (magenta). Overlapping signals appear white (Merge). (D') Limited overlap was observed between vinculin (green) and ALP-1 (magenta). Arrows indicate dense plaques, structures found at muscle–muscle junctions. Note the absence of ALP-1 at muscle–muscle junction (arrow) in 2D. Bar, 10 μm. (E) Schematic diagram of the components of a *C. elegans* dense body.

Characterization of the alp-1 Mutants

To explore the role of ALP-1 in muscle dense bodies, we requested the production of *alp-1* deletion alleles from the National Bioresource project in Japan and received the deletion allele *alp-1(tm1137)*. Another allele, *alp-1(ok820)*, was generated by the *C. elegans* Gene Knockout Consortium (Oklahoma City, OK). We confirmed that the *alp-1(ok820)* allele contains a deletion from 2804 to 4039 bp, covering the region in the intron between exons 5 and 6 as well as part of exon 6 (Figure 1A), consistent with the deletion report from the knockout consortium. According to the predicted gene structure (McKeown *et al.*, 2006), this is an in-frame deletion that would result in a 26-amino acid deletion that does not affect the LIM1 domain (Figure 1B). The other deletion allele, *alp-1(tm1137)*, was initially reported (Japanese National Bioresource Project) to carry a deletion from position 3993–4398 bp as well as an additional 3-bp insertion. However, sequencing of this allele in our laboratory reproducibly showed that *alp-1(tm1137)* is deleted from 3995 to 4398 bp (Figure 1A), which is predicted to eliminate the entire LIM1 domain and resulting in a translational frameshift (Figure 1B).

To visualize the impact of the *alp-1(ok820)* and *alp-1(tm1137)* deletions on the ALP-1 protein products, we performed Western analysis using the B74 and B78 anti-ALP-1 antisera on deletion mutants (Figure 4). Because both the *alp-1(ok820)* and *alp-1(tm1137)* deletions would affect coding sequences shared by all isoforms, these mutations are assumed to affect all four resulting proteins (Figure 1, A and B). Using either the B74 or B78 antibody, Western immunoblot results showed that *alp-1(ok820)* mutants produced ALP-1 isoforms with altered, faster mobility, consistent with the prediction that the *alp-1(ok820)* deletion results in truncated ALP-1 protein products (Figure 4). In contrast, *alp-1(tm1137)* mutants produced no detectable ALP-1A, B, and D isoforms by Western immunoblot analysis (Figure 4). The nature of the *alp-1(tm1137)* mutation suggests that ALP-1C, if it does exist, should also be affected by the deletion, therefore *alp-1(tm1137)* is likely to be a molecular null.

alp-1 Mutants Are Viable and Display Normal Muscle Function

Homozygous *alp-1* mutants are viable and produce a similar number of progeny as wild type (Figure 5A), indicating that *alp-1* is not an essential gene. In addition, *alp-1* mutants displayed no gross morphological defects (data not shown). Because ALP-1 proteins are expressed in the pharynx and body wall muscle, we examined muscle function in *alp-1* mutants in two ways: 1) a pumping assay to measure the contractility of pharyngeal muscles and 2) a thrashing assay to test the contractility of body wall muscle. Surprisingly, the *alp-1(tm1137)* mutants displayed muscle contractility that was indistinguishable from wild type (Figure 5A), indicating that *alp-1* mutants have normal pharyngeal and body wall muscle function.

To complement the mutant analysis, we also used an RNAi strategy to examine the function of the *alp-1* gene. RNA-mediated interference by feeding has been used to inactivate specific genes in *C. elegans* (Kamath *et al.*, 2001). The RNAi clone IV-4D13 (*alp-1* RNAi) from an established *C. elegans* RNAi library (Kamath and Ahringer, 2003) is predicted to knockdown all *alp-1* transcripts (Figure 1A; see *Materials and Methods*). Western immunoblot analysis using the anti-ALP-1 B78 antibody showed that the levels of ALP-1 proteins are substantially reduced in N2(*alp-1 RNAi*) (Figure 5B). Indirect immunofluorescence experiments showed that

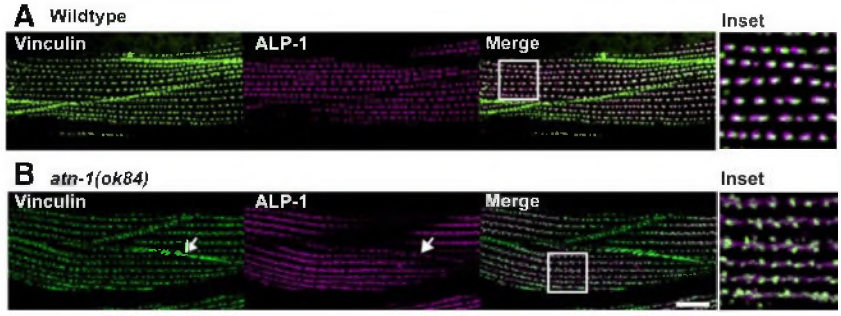

Figure 3. Proper localization of ALP-1 in dense bodies is dependent on α-actinin. (A and B) Dense bodies in *α-actinin* mutants are less well defined, as shown by vinculin staining, but still show a periodic punctate appearance. In *α-actinin* mutants, ALP-1 staining is abnormal, appearing continuous, rather than as a dotted line. Sometimes ALP-1 was mislocalized to dense plaques (muscle–muscle junctions), as indicated by arrows. Vinculin (green); ALP-1 (magenta). Bar, 10 μm.

ALP-1 proteins were below detectable levels in the *alp-1* RNAi-treated worms (Figure 5C). In the pumping and thrashing assays, RNAi inactivation of the *alp-1* gene did not result in any functional failure in muscle (Figure 5A), consistent with the results from *alp-1(tm1137)* mutants. To achieve maximal elimination of ALP-1 products, we performed RNAi knockdown (*alp-1* RNAi) on *alp-1* mutants. Any residual ALP-1 proteins that may not be able to be detected by Western blot analysis would be eliminated in the *alp-1(alp-1 RNAi)* animals. *alp-1(alp-1 RNAi)* animals were viable and fertile and showed similar muscle contractility to *alp-1(control RNAi)* animals in the pharyngeal pumping and thrashing assays (data not shown).

alp-1 Mutants Display Defects in Actin Filament Organization in Muscle Cells

Although the *alp-1(tm1137)* mutants have grossly normal muscle function, it was still possible that the mutants could have disturbed muscle structure that does not impact function significantly. Thus, we examined the muscle architecture of the *alp-1* mutants for any structural defects. As shown in Figure 6, both α-actinin and vinculin were deposited normally at dense bodies, and myosin displayed an undisturbed striated pattern in *alp-1* mutants. Interestingly, examination of F-actin organization revealed that a small percentage of *alp-1* mutants exhibited an alteration in the actin filaments of the body wall muscle (see Figure 7B). Specifically, small actin aggregates at the ends of muscle cells were observed in *alp-1* mutants. Although this actin aggregation phenotype was found in only a small percentage of *alp-1* mutants (8.8%; n = 272), it was reproducibly observed and never found in wild-type worms (0%; n = 201; Figure 7C). The actin aggregation phenotype in *alp-1(tm1137)* mutants was rescued by introducing *alp-1* transgene (0%; n = 217; Figure 7C).

The presence of actin aggregates in *alp-1* mutants suggests that the structural integrity of actin filaments is compromised in the absence of ALP-1. We were intrigued to test whether this actin phenotype is associated with contractile activity. It is known that treatment of *C. elegans* with the acetylcholine receptor agonist, tetramisol, induces muscle hypercontraction, placing strain on the myofilament lattice (Brenner, 1974; Ono *et al.*, 2006). Thus, we treated *alp-1*

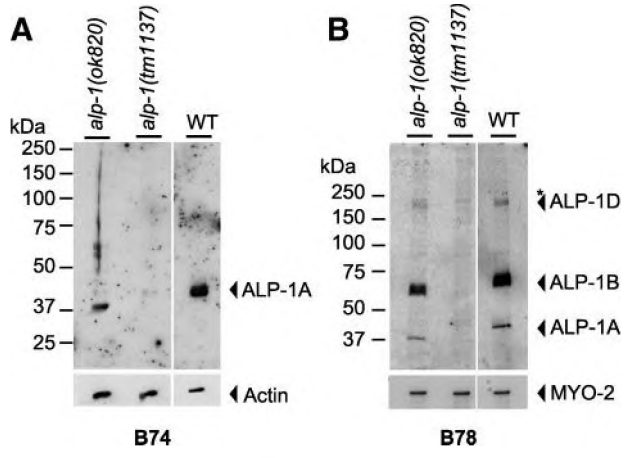

Figure 4. The Western analysis of *alp-1* mutants. Using the (A) B74 anti-ALP-1A–specific antibody and (B) B78 anti-ALP-1PDZ antibody, ALP-1 proteins displayed altered mobility *in alp-1(ok820)* mutants and were absent in *alp-1(tm1137)* mutants on Western immunoblots. Anti-actin and anti-myo2 antibodies were used as loading controls. The asterisk indicates a background band just above ALP-1D. This background band is observed even when the ALP-1 proteins fail to be detected in peptide antigen competition experiment.

A

Genotype	Brood Size (22 °C; $n > 10$)	Pumping Assay ($n \geq 15$)	Thrashing Assay ($n \geq 15$)
Wild-type N_2	278.2 ± 10.6	27.1 ± 0.3	94.8 ± 1.7
alp-1(ok820)	299.0 ± 9.9 ($p = 0.17$)*	n. d.	n. d.
alp-1(tm1137)	294.4 ± 12.9 ($p = 0.34$)*	26.5 ± 0.3 ($p = 0.23$)*	98.9 ± 1.4 ($p = 0.07$)*
N_2*(control RNAi)*	n. d.	27.1 ± 0.4	99.2 ± 1.8
N_2*(alp-1 RNAi)*	n. d.	27.5 ± 0.3 ($p = 0.39$)†	99.7 ± 1.7 ($p = 0.85$)†

average ± s.e.m; n.d. = not determined
* = *p*-value represents comparison to wild-type N2
† = *p*-value represents comparison to N_2*(control RNAi)*

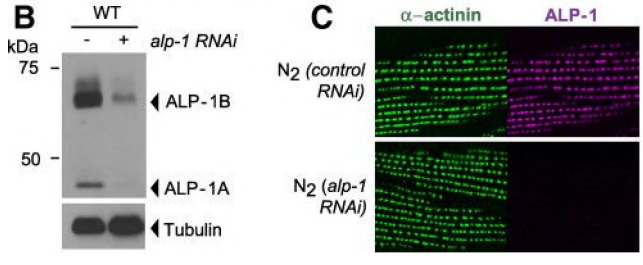

Figure 5. Loss of ALP-1 proteins in *alp-1* mutants or N2(*alp-1 RNAi*) worms does not alter muscle function. (A) Quantification of functional assays reveals the *alp-1* gene is not essential, and no statistical difference in muscle contractility between wild-type N2 and *alp-1* mutants, and N2(*control RNAi*) and N2(*alp-1 RNAi*) animals. (B) Western immunoblot analysis shows a knockdown of ALP-1 proteins in N2(*alp-1RNAi*) worms. (C) In indirect immunofluorescence experiments, ALP-1 proteins are below detectable levels in N2(*alp-1RNAi*) animals. α-actinin, green; ALP-1, magenta.

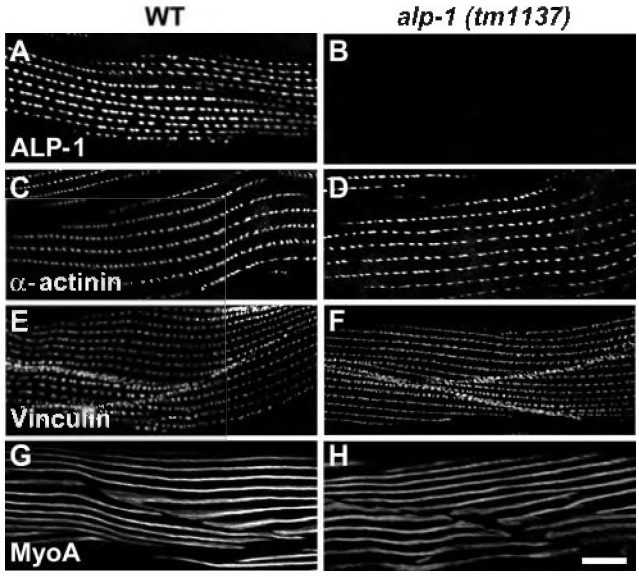

Figure 6. Dense bodies and myosin filaments appear normal in *alp-1(tm1137)* mutants. The body wall muscle from wild-type worms (A, C, E, and G) and *alp-1(tm1137)* mutants (B, D, F, and H) are stained for (A and B) ALP-1 protein (B78 anti-ALP-1PDZ antibody). (C and D) α-actinin; (E and F) vinculin; (G and H) myosin filaments (anti-MyoA antibody). Bar, 10 μm.

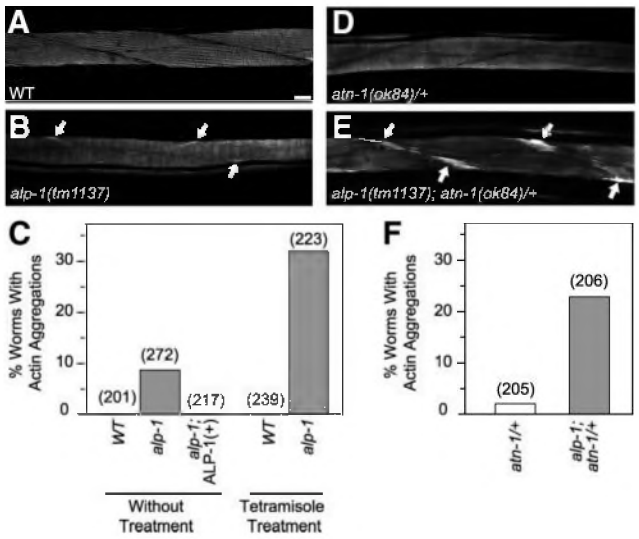

Figure 7. ALP-1 and α-actinin function together to stabilize actin filament organization. (A and B) *alp-1* mutants display an actin-aggregation phenotype at the ends of muscle cells (arrows). Representative phalloidin-stained images from WT worms (A) and *alp-1(tm1137)* mutants (B). (C) Hypercontraction, induced by tetramisole treatment increases the penetrance of the actin aggregation phenotype displayed in *alp-1* mutants. (D and E) Reduction of α-actinin activity enhances the actin-filament organization phenotype caused by loss of ALP-1. Representative phalloidin-stained images from worms heterozygous for an *atn-1(ok84)* mutation (D) and worms homozygous for the *alp-1(tm1137)* mutation and heterozygous for the *atn-1(ok84)* mutation (E). Arrows indicate actin-aggregation at the ends of muscle cells. (F) The penetrance of the actin-aggregation phenotype in *alp-1* mutants is increased by the reduction of α-actinin activity. Bar, 10 μm. The number in parentheses indicates the number of worms examined. $p = < 0.001$ in each set of the experiments by chi-square analysis.

mutants with tetramisole to see whether an increased load on the muscles exacerbate the actin-aggregation phenotype. Here, we found that tetramisole treatment enhanced the observed actin-aggregation phenotype about fourfold (31.8%; n = 223) relative to untreated *alp-1* mutant worms (Figure 7C). This result suggests that ALP-1 plays a role in stabilizing the actin myofilament lattice during times of increased muscle load.

ALP-1 Functions Together with α-Actinin to Maintain Actin Filament Integrity in Muscle Cells

Our observation that the *alp-1* mutants exhibited a minor disturbance in muscle structure raises the possibility that other genes may participate in the same biological process and compensate for the loss of ALP-1. α-actinin is a major contributor to actin filament organization and has been identified as a high confident interaction partner of ALP-1 in a *C. elegans* genome-wide yeast-two hybrid screen (Li *et al.*, 2004). ALP-1 is colocalized with α-actinin at dense bodies in muscles (Figure 2C), and the α-actinin mutants, *atn-1*, also display actin aggregation phenotype although the phenotype is more severe than that observed in *alp-1(tm1137)* mutant worms (Figure 7, B and C; Ono *et al.*, 2006). These data indicate that α-actinin and ALP-1 may act together to maintain actin cytoarchitecture integrity. To directly test this idea, we looked for enhancement of the actin aggregation phenotype in *alp-1* mutants under conditions of reduced α-actinin activity. If ALP-1 facilitates α-actinin's ability to stabilize actin filament organization, then reduction of wild-type α-actinin gene products should enhance the actin aggregation phenotype seen in the *alp-1* mutants. We generated the strain *alp-1(tm1137); atn-1(ok84)/+*, which carries a homozygous *alp-1(tm1137)* mutation and a heterozygous *atn-1(ok84)* mutation. Although animals heterozygous for the *atn-1* gene have no evident defect in actin filament organization on their own (Figure 7D), loss of ALP-1 activity in the α-actinin heterozygous mutant background results in profound enhancement of the incidence of actin aggregation in the body wall muscle as compared with *alp-1(tm1137)* alone (Figure 7, E and F), suggesting that ALP-1 and α-actinin function together to maintain actin filament integrity. We also generated *alp-1* and *α-actinin* double mutants and examined their body wall muscle for evidence of enhanced actin aggregation. We found that the actin filament phenotype in homozygous *alp-1(tm1137); atn-1(ok84)* double mutants was equivalent to that seen in the *atn-1(ok84)* animals (data not shown). Because both *alp-1(tm1137)* and *atn-1(ok84)* are thought to be null alleles, the lack of an additive effect on the actin aggregation phenotype in the double mutants suggests that *alp-1* and *atn-1* are not functioning in parallel processes that stabilize actin filaments. Collectively, these genetic results indicate that ALP-1 functions together with α-actinin to maintain actin filament stabilization.

alp-1 and ketn-1 Genetically Interact to Provide Mechanical Stability for Normal Muscle Function

The *ketn-1* gene encodes the *C. elegans* kettin protein, a member of the connectin/titin gene family (Bullard *et al.*, 2002; Ono *et al.*, 2006). A previous report showed that the product of the *ketn-1* gene functions in concert with α-actinin to stabilize actin filament organization in *C. elegans* (Ono *et al.*, 2006). To investigate whether *alp-1* genetically interacts with *ketn-1*, we examined the effect of the reduction of kettin activity on actin filament organization in *alp-1* mutants (Figure 8). In a wild-type background, RNAi knockdown of the *ketn-1* gene induced actin-aggregation at the end of muscle

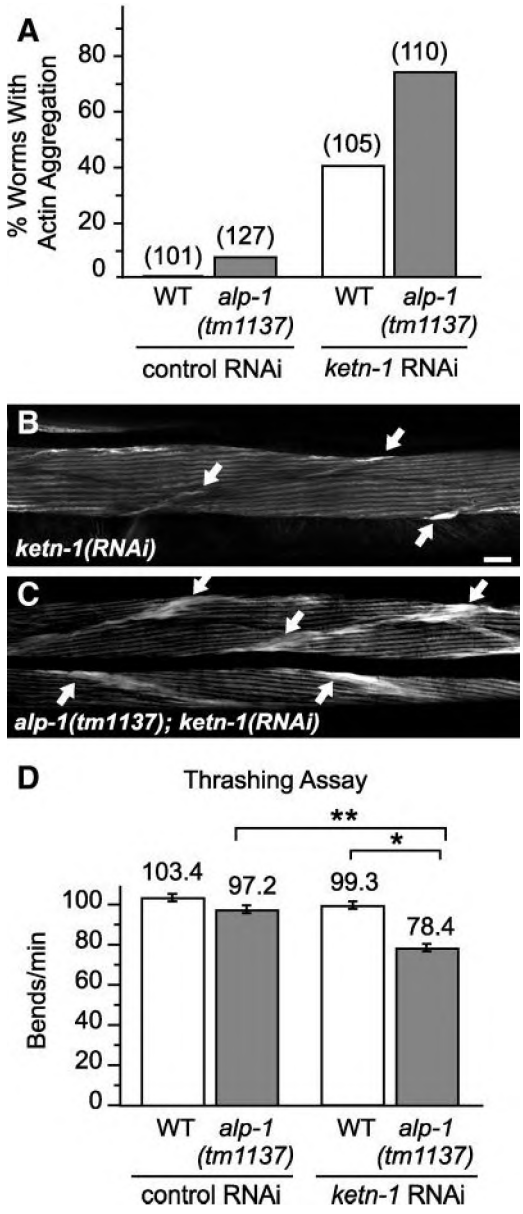

Figure 8. *alp-1* genetically interacts with *ketn-1* to provide mechanical stability for normal muscle function. (A) The actin aggregation phenotype observed in *alp-1(tm1137)* mutants is dramatically enhanced in the *ketn-1(RNAi)* background. (B and C) Phalloidin staining of actin filaments in *ketn-1(RNAi)* (B) and *alp-1(tm1137);ketn-1(RNAi)* (C). Arrows indicate actin-aggregation at the ends of muscle cells. Bar, 10 μm. (D) Impaired body wall muscle contractility is observed in the thrashing assay for *alp-1(tm1137);ketn-1(RNAi)* relative to *ketn-1(RNAi)*, (*p = 7.56E-11) and *alp-1(tm1137); ketn-1(RNAi)* relative to *alp-1(tm1137); control RNAi*, (**p = 1.03E-09).

cells at low penetrance as shown in Figure 8A, and as previous reported (Ono *et al.*, 2006). In the *alp-1(tm1137)* mutant background, *ketn-1(RNAi)* displayed significant enhancement of actin-aggregation phenotype (73.6%; n = 110) as compared with either *ketn-1(RNAi)* alone (40%; n = 105) or *alp-1(tm137); control RNAi* (7.1%; n = 127; Figure 8A). In addition, the actin filaments were more disorganized with much larger aggregates when *ketn-1* RNAi was performed in the *alp-1(tm1137)* background (Figure 8C). We next performed a thrashing assay to determine whether the contractility of body wall muscle is impaired when actin filament organization is severely disturbed. In the thrashing assay, we showed that the strong actin-aggregation phenotype in *alp-1(tm1137);ketn-1(RNAi)* animals is associated with impaired muscle function (Figure 8D). Our data demonstrate a genetic interaction between *alp-1* and *ketn-1* that is critical for both actin filament stabilization and normal muscle function.

DISCUSSION

We used a genetic approach in *C. elegans* to clarify the molecular function of ALP-Enigma proteins in vivo. We characterized *alp-1* mutants and showed that ALP-1 functions to stabilize actin filament organization in body wall muscle cells. Reduction of α-actinin or kettin in the *alp-1* mutants results in increased destabilization of the actin filaments and is associated with impaired contractility of body wall muscles. Our data reveal a possible mechanism for how ALP-Enigma proteins affect muscle function, and propose that ALP-1 and α-actinin function together to stabilize the architecture of the contractile apparatus.

ALP-1 Is a Muscle Protein and Functions Primarily in Body Wall Muscle in C. elegans

In this report, we generated anti-ALP-1 antibodies which specifically recognize *alp-1* gene products by Western analysis and indirect immunostaining. We determined that endogenous ALP-1 proteins are highly expressed in the body wall muscle, where they are localized to the dense bodies, specialized actin anchorage sites, consistent with our previous report using a GFP translational reporter (McKeown *et al.*, 2006). ALP-1::GFP was also reported to localize to body wall muscle nuclei, however we did not observe nuclear staining using anti-ALP-1 antibodies. The addition of a GFP tag, which increases the molecular weight of the ALP-1 proteins, may increase the nuclear dwell time of the fusion protein, enabling its detection in the nuclear compartment. Alternatively, the accumulation of ALP-1::GFP may not accurately reflect the distribution of native ALP-1.

The pharynx is a musculature feeding structure in the worm. Not surprisingly, ALP-1 is expressed in this organ. Endogenous ALP-1 proteins are detected in the pharynx by B78 (against ALP-1A, B, and D) but not B74 (against ALP-1A), suggesting one or more enigma isoforms (ALP-1B, C, and D) are responsible for this pharyngeal staining, and revealing distinct expression pattern for enigma and ALP isoforms.

Characterization of alp-1 Mutants in C. elegans

In mammals, ablation the ALP-Enigma family members, Alp or Cypher, results in cardiac defects and skeletal muscle pathology, respectively (Pashmforoush *et al.*, 2001; Zhou *et al.*, 2001). Depletion of ALP-Enigma proteins in *Drosophila* has recently been reported to cause Z-line defects and first instar larvae lethality (Jani and Schock, 2007). Surprisingly, our analysis showed that *alp-1*, the sole gene for the entire ALP-Enigma family in *C. elegans*, is not essential. The *alp-1(tm1137)* deletion results in the apparently complete loss of the ALP-1A, B, and D isoforms by Western immunoblot analysis and the nature of the mutation predicts similar impact on the hypothetical protein product, ALP-1C, suggesting *alp-1(tm1137)* is a null allele. In support of this conclusion, we performed RNAi to knockdown any residual *alp-1* gene products in *alp-1* mutants. The *alp-1(alp-1 RNAi)* did not produce a more severe phenotype than *alp-1(control*

RNAi) worms, further reinforcing the conclusion that *alp-1(tm1173)* is a null, and ALP-1 is not essential. There are several examples of muscle gene defects that result in lethality in *Drosophila* but produce much more subtle phenotypes in *C. elegans* (Moerman *et al.*, 1982; Bessou *et al.*, 1998; Flaherty *et al.*, 2002). One simple explanation is the existence of *C. elegans*-specific proteins redundant for muscle function. Another explanation might be that albeit the basic sarcomere structure is similar, there is some difference in sarcomere arrangement and organization in *C. elegans* from flies and vertebrates. For example, the nematode muscle is obliquely striated (the adjacent sarcomeres are staggered) whereas the vertebrate muscle is cross-striated (the adjacent sarcomeres are aligned; Lecroisey *et al.*, 2007). These may result in different muscle load or stress accumulation in the muscle cells and lead to the difference of observed phenotype. The viable phenotype of *alp-1(tm1137)* mutants has enabled us to explore ALP-1 mechanism of action via genetic interaction studies.

The Relationship between ALP-1 and α-Actinin

In our studies, using available genetic mutants and reagents, we evaluated the relationship between ALP-1 and α-actinin in vivo. We found that ALP-1 and α-actinin are colocalized at dense bodies. In addition, α-actinin is required for ALP-1 targeting to dense bodies. We further showed by genetic interaction studies that ALP-1 and α-actinin function together to stabilize the muscle contractile apparatus, in particular the actin-rich thin filaments. As α-actinin acts to anchor actin filaments at Z-discs (Maruyama and Ebashi, 1965; Blanchard *et al.*, 1989), our data propose a model that α-actinin recruits ALP-1 to dense bodies where ALP-1 participates in α-actinin–dependent anchorage and stabilization of the actin myofiliments within muscle cells. A previous in vitro study showing that Alp enhances α-actinin-dependent bundling of actin filaments (Pashmforoush *et al.*, 2001) is in support of this model. Notably, since in the absence of α-actinin, ALP-1 seems to retain its ability to associate with actin filaments, another ALP-1 protein partner must be sufficient to recruit ALP-1 to the actin cytoskeleton. To date, there is no evidence that ALP-1 interacts directly with actin, therefore an as-yet-unidentified protein must be responsible for tethering ALP-1 to actin filaments.

Mammalian studies have indicated that α-actinin is a prominent ALP-Enigma binding partner (Xia *et al.*, 1997; Faulkner *et al.*, 1999; Pomies *et al.*, 1999; Zhou *et al.*, 1999; Kotaka *et al.*, 2000; Nakagawa *et al.*, 2000; Niederlander *et al.*, 2004; Schulz *et al.*, 2004). Importantly, α-actinin was also identified with high confidence as an interaction partner of ALP-1 in *C. elegans* genome-wide yeast-two hybrid screens (Li *et al.*, 2004). These data suggest that the recruitment of ALP-1 to dense bodies may depend on direct ALP-1–ATN-1 interaction which is consistent with our results that ATN-1 is required for the proper localization of ALP-1. However, so far we cannot demonstrate the ALP-1–ATN-1 interaction by coimmunoprecipitation in worm lysates. It remains a formal possibility that ALP-1 is dependent on ATN-1 for its proper localization and this dependence is unrelated to their direct interaction.

A Link between alp-1 and a Connectin/Titin Family Member, Kettin

kettin is a titin/connectin family member found in the Z-discs and the I-bands of invertebrate muscles (Maki *et al.*, 1995; Hakeda *et al.*, 2000; Bullard *et al.*, 2002; Ono *et al.*, 2005). This protein has been shown to be essential for the integrity of the Z-disk (Lakey *et al.*, 1993) and directly associates with actin (Lakey *et al.*, 1993; van Straaten *et al.*, 1999; Ono *et al.*, 2006). In *C. elegans*, kettin genetically interacts with α-actinin to maintain actin filament organization (Ono *et al.*, 2006). We demonstrated that *alp-1* genetically interacts with kettin to provide myofibril stability. A direct physical interaction between Ce-kettin and α-actinin has not been established in the worm, however titin/connectin proteins have been shown to directly interact with α-actinin in vertebrates (Ohtsuka *et al.*, 1997a,b; Sorimachi *et al.*, 1997; Young *et al.*, 1998). Moreover, structural analysis has suggested the possibility of a titin/α-actinin/zasp (Enigma family member) ternary complex (Au *et al.*, 2004). Likewise, ALP-1, α-actinin, and kettin may form a ternary complex. Our genetic interaction data support this model and suggest that this protein complex functions to stabilize the actin myofilaments to maintain muscle structural integrity.

A Role for ALP-1 in Stabilizing Actin Filaments

The *alp-1* mutants display actin-aggregates at the end of muscle cells. We showed that hypercontraction induced by tetramisole enhanced this actin aggregation defect. A similar actin phenotype in *ketn-1* mutants has also been reported, and it was shown that this actin defect could be suppressed by *unc-54* background. The *unc-54* mutants contain a myosin heavy chain mutation and display reduced muscle contraction (Ono *et al.*, 2006). Therefore, these results suggest that this actin aggregation phenotype is sensitive to the state of muscle contraction and the severity is correlated with muscle loading. The actin aggregates may stem from the detachment of actin filaments from dense bodies or the dissolution of actin arrays in muscle cells. Our data favor the former interpretation since ALP-1 localizes at dense bodies, which are actin-filament anchorage sites, and we showed that ALP-1 functions together with α-actinin, a predominant actin cross-linking protein. This observed actin filament phenotype in *alp-1*mutants indicates that the disruption of actin myofilament integrity might be the primary event triggering compromised muscle function in the loss of ALP-Enigma proteins in vertebrates. Intriguingly, mutations in the gene *unc-87*, which encodes a calponin-related protein, also result in actin aggregation within muscle cells (Goetinck and Waterston, 1994a,b). Ono and colleagues have characterized UNC-87 protein and showed that it antagonizes actin depolymerization factor (ADF)/cofilin-mediated actin filament turnover, suggesting a critical role for actin dynamics in the maintenance of actin architecture in muscle (Yamashiro *et al.*, 2007). Thus, the actin anchorage proteins, such as α-actinin and ALP-1, and the actin dynamic regulatory proteins UNC-87 and UNC-60B (Ce-ADF) might represent two parallel pathways that participate in stabilizing muscle myofilaments.

Recently, human mutations in Cypher have been linked to dilated cardiomyopathy and muscular dystrophy in patients (Vatta *et al.*, 2003; Arimura *et al.*, 2004; Selcen and Engel, 2005). These findings highlight the important role of ALP-Enigma proteins in muscle maintenance and illustrate the need for increased understanding of the molecular mechanisms by which this family of PDZ-LIM proteins contributes to muscle structure and function. Our analysis of ALP-Enigma function in *C. elegans* and the demonstration of genetic interactions between *alp-1* and both α-actinin and kettin provide new insights into the role of ALP-Enigma proteins in molecular pathology, and enhance our knowledge of how muscle cells provide mechanical stability for supporting normal muscle contractility.

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